Amendments to the Specification:

Please replace the original paper copy of the Sequence Listing with the substitute paper copy of the Sequence Listing filed herewith.

At page 1, line 1, please delete subheading:

~~DESCRIPTION~~

Please amend the title to read as:

METHODS OF SCREENING FOR AGONISTIC ANTIBODIES

Please replace the paragraph beginning at page 1, line 18 with the following amended paragraph:

In many cytokine receptors, it is thought that the angle and length of chains that form homo/hetero-dimers change when a ligand binds, thus enabling the receptors to transmit signals into cells. Thus, appropriate anti-receptor antibodies can mimic receptor dimerization initiated by ligand-binding, and become potential agonistic antibodies. Monoclonal antibodies that display agonistic ~~function~~ activity against MPL, a homodimer, have already been reported (Blood 1998 Sep 15; 92(6): 1981-8, US98/17364). However, to obtain such agonistic antibodies, selection must be made from a huge range of antibodies, requiring effective selection methods.

Please replace the paragraph beginning at page 1, line 28 with the following amended paragraph:

In conventional assays, it is necessary to select antibodies that bind to antigens, *i.e.*, receptor chains, and add these antibodies to an appropriate cell assay system that responds to the ligands. This becomes particularly troublesome where the receptors form heterodimers. Antibodies against each of the two chains (A, B) that form the receptor must be selected, and every combination of A and B must be tested one by one. In addition, to generate bivalent antibodies, it is necessary to fuse antibody-producing hybridomas, or to construct expression

vectors for all of the antibodies, and introduce all combinations thereof into cells. Examination of 100 types of antibodies against each of the A and B chains necessitates the testing of 10,000 types of combinations, and requires a total of 400 types of expression vectors for L and H chains to be constructed and introduced into cells 10,000 times. There are also methods that use libraries that provide antibodies displayed on phages as bispecific diabodies. However, since the direct addition of *E. coli* culture supernatant to cell culture systems has ~~an impact~~ a bad effect on cells, purification becomes necessary and monospecific diabody contamination (theoretically 50%) becomes inevitable.

Please replace the paragraph beginning at page 2, line 36 with the following amended paragraph:

Mice are first immunized with either A-chain or B-chain receptors, and mRNAs are extracted from the splenocytes of these animals. L-chain and H-chain variable regions are recovered by RT-PCR using primers that correspond to mouse CDRs. Single chain variable regions (scFvs) are synthesized by assembly PCR to construct a phage library. Antigen-binding antibody clones are concentrated by panning, the synthesized single chain variable regions obtained from concentrated clones are inserted between a signal sequence for animal cells and CH1-hinge-CH2-CH3 and, to construct a library that is integrated into plasmids to be used for producing retroviruses. By expressing chimeric chains comprised of a target receptor chain and the G-CSFR intracellular domain, Ba/F3 cells, whose proliferation depends on the binding of the ligand to target chimera receptor, are prepared. These cells are infected with anti-A-chain antibody library viruses. These cells are further infected with anti-B-chain antibody library viruses, and cultured following the washing and removal of ~~factors~~ ligands (factors). Cells that now reproduce ~~factor~~ ligand (factor)-dependently are recovered and cloned, and agonistic activity is confirmed by using the culture supernatants and physiological assay systems. The antibody CDR genes incorporated in the chromosomes of the clones are recovered using PCR, and applied to the production of multi-specific agonistic antibodies. In this method, effective

screening can be carried out using antibodies as libraries. Furthermore, the manipulations are simple, and there is no need for complicated operations.

Please replace the paragraph beginning at page 3, line 27 with the following amended paragraph:

In other words, the present invention relates to novel methods that can effectively screen for multi-specific agonistic antibodies. More specifically, the present invention provides:

[1] a method of screening for agonistic antibodies that comprises the following steps (a) to (c):

(a) providing a cell that expresses a multimer-forming receptor and a test antibody, where the cell grow depending on[~~a~~] the corresponding ligand of the receptor~~factor~~;

(b) determining the test antibody to comprise agonistic~~function~~ activity when autocrine cell growth is autonomous; and

(c) selecting those antibodies that comprise agonistic~~function~~ activity;

[2] the method of [1] that further comprises the step of introducing a gene that encodes the heavy chain of the test antibody into the cell of step (a) having been introduced with a gene that encodes the light chain of the test antibody and a gene that encodes the receptor;

[3] the method of [1] or [2] where the receptor is a chimeric receptor with a protein that comprises a function of transducing a cell growth signal;

[4] the method of any one of [1] to [3] where the receptor is a dimer-forming receptor;

[5] the method of [4] where the dimer-forming receptor is a homo-dimer;

[6] the method of [4] where the dimer-forming receptor is a hetero-dimer;

[7] the method of any one of [1] to [6] where the protein that comprises the function of transducing a cell growth signal is a G-CSF receptor;

[8] the method of any one of [1] to [7] that comprises the introduction of an antibody library to the cell;

[9] the method of [8] where the antibody library is a retroviral antibody library;

[10] the method of any one of [1] to [9] where the test antibody is a multi-specific antibody;

[11] the method of [10] that comprises linking the test antibody's heavy and light chain variable regions with a linker;

[12] the method of [11] that comprises producing the antibody with variable regions linked by a linker, using a method that comprises the steps (a) to (c):

(a) producing a single chain Fv against the first receptor chain;

(b) producing a single chain antibody against the first receptor chain by linking the single chain Fv with a CH1-hinge-CH2-CH3; and

(c) producing a multi-specific antibody that comprises the single chain antibody produced in step (b);

[13] the method of [11] that comprises producing the antibody with its variable regions linked by a linker, using a method that comprises the steps (a) to (c):

(a) producing a single chain Fab against the first receptor chain;

(b) producing a single chain antibody against the first receptor chain by linking the single chain Fab with an Fc; and

(c) producing a multi-specific antibody that comprises the single chain antibody produced in step (b);

[14] a method of screening for an agonist multi-specific antibody that comprises the steps (a) to (c):

(a) contacting between a multi-specific antibody and a receptor comprising a first receptor chain and a second receptor chain, where the multi-specific antibody comprises a variable region that can bind with the first receptor chain and a variable region that can bind with the second receptor chain;

(b) determining whether the test multi-specific antibody comprises agonistic ~~function~~ activity; and

(c) selecting antibodies that comprise agonistic ~~function~~ activity;

[15] the method of [14] that comprises expressing the receptor and the test multi-specific antibody in the same cell;

[16] the method of [15] where the cell is a cell that grows depending on[~~a~~] the corresponding ligand of the receptor;

[17] the method of [15] or [16] where the receptor comprises the function of transducing a cell growth signal;

[18] the method of [17] where the receptor is a chimeric receptor with a protein that comprises the function of transducing a cell growth signal;

[19] the method of [18] where the protein that comprises the function of transducing a cell growth signal is a G-CSF receptor;

[20] the method of any one of [15] to [19] where the test multi-specific antibody is determined to comprise agonistic ~~function~~ activity when autocrine cell growth is autonomous;

[21] the method of any one of [15] to [20] that further comprises the step of introducing an antibody library against the first receptor chain and the second receptor chain into the cell, respectively;

[22] the method of [21] where the antibody library is a retroviral antibody library;

[23] the method of any one of [14] to [22] that comprises linking the light chain variable regions and heavy chain variable regions of the multi-specific antibody with a linker;

[24] the method of [23] that comprises producing a multi-specific antibody with variable regions linked by a linker, using a method that comprises steps (a) to (c):

(a) producing a single chain Fv against the first receptor chain;

(b) producing a single chain antibody against the first receptor chain by linking the single chain Fv with a CH1-hinge-CH2-CH3; and

(c) producing a multi-specific antibody that comprises the single chain antibody produced in step (b);

[25] the method of [23] that comprises producing the multi-specific antibody with variable regions linked by a linker, using a method that comprises steps (a) to (c):

(a) producing a single chain Fab against the first receptor chain;

(b) producing a single chain antibody against the first receptor chain by linking the single chain Fab with an Fc; and

(c) producing a multi-specific antibody that comprises the single chain antibody produced in step (b);

[26] the method of any one of [14] to [25] that comprises the introduction of "Knobs-into-holes" by amino acid substitution at the CH3 region of the multi-specific antibody~~[CH3]~~;

[27] the method of any one of [14] to [26] where the multimer of the receptor is a heterodimer;

[28] the method of any one of [14] to [27] where the multi-specific antibody is a bispecific antibody;

[29] a method for producing an agonistic antibody comprising steps (a) to (c):

(a) screening for an agonistic antibody by a method of any one of [1] to [28];

(b) introducing a gene that encodes the agonistic antibody selected by the screening of step (a) into a host cell;

(c) recovering the agonistic antibody from the host cell of step (b) or its cell culture supernatant;

[30] a cell that expresses an antibody, and a receptor that multimerizes by binding with the antibody, where the cell grow depending on ~~a factor~~ the corresponding ligand of the receptor;

[31] the cell of [30] where the receptor is a chimeric receptor with a protein that comprises the function of transducing a cell growth signal;

[32] the cell of [30] or [31] where the antibody is a multi-specific antibody;

[33] the cell of any one of [30] to [32] where the receptor that is multimerized by binding with the antibody comprises the function of transducing a cell growth signal;

[34] a multi-specific agonistic antibody that comprises the linking of the light chain variable region and heavy chain variable region by linkers, and the introduction of "Knobs-into-holes" by amino acid substitution at the CH3 region of the antibody[CH3].

Please replace the paragraph beginning at page 7, line 25 with the following amended paragraph:

"Agonistic antibody" refers to an antibody that comprises an agonistic ~~function~~ activity against a given receptor. In general, when an agonist ligand (factor) binds to a receptor, the tertiary structure of the receptor protein changes, and the receptor is activated (when the receptor is a membrane protein, a cell growth signal or such is usually transducted). If the receptor is a dimer-forming type, an agonistic antibody can dimerize the receptor at an appropriate distance and angle, thus acting similarly to a ligand. An appropriate anti-receptor antibody can mimic dimerization of receptors performed by ligands, and thus can become an agonistic antibody.

Please replace the paragraph beginning at page 11, line 11 with the following amended paragraph:

The above-mentioned cells of the present invention are usually eukaryote-derived cells, preferably animal cells, and more preferably human-derived cells. In a preferable embodiment of the present invention, cells expressing test antibodies also express the above receptors (the receptors for which agonistic antibodies act as agonists). Thus, a preferable embodiment of the present invention comprises expressing a receptor and a test antibody in the same cell. If a test antibody secreted from a cell binds with that receptor and comprises agonistic ~~function~~ activity against a receptor, the receptor would transduce a cell growth signal and consequently, the cell would undergo autonomous autocrine replication. "Autonomous autocrine replication" refers to the phenomenon whereby cells replicate independently using a substance produced by the cell itself as a growth signal. Multi-specific agonistic antibodies can be screened using the presence or absence of autonoumous autocrine replication as an index. In a preferable embodiment of the present invention, when cells expressing a test antibody and receptor undergo autonomous autocrine replication, the test antibody can be determined to comprise agonistic ~~function~~ activity.

Please replace the paragraph beginning at page 11, line 24 with the following amended paragraph:

Agonistic ~~function~~ activity of the test antibodies of the present invention can be determined using the indexes below:

(1) Whether or not a ligand (factor)-dependently growing cell will grow in the same way when a test antibody is added during cell culture as when a ligand (factor) is added. If the cell grows, the test antibody is determined to comprise agonistic ~~function~~ activity.

(2) Whether or not a cell line with intrinsic ligand (factor)-dependent activities (not limited to growth) shows the same reaction when a test antibody is added during cell culture as when a ligand (factor) is added. If the cell line shows the same reaction as for a ligand (factor), the test antibody is determined to comprise agonistic ~~function~~ activity.

Please replace the paragraph beginning at page 11, line 36 with the following amended paragraph:

In the present invention, cells transducing the above-mentioned cell growth signals usually express the receptors for which the antibodies selected by the screening methods of the present invention act as agonists on the cell surface. These cells transduce cell growth signals by binding with the ligands of those receptors (for example, agonistic antibodies). Thus, in the present invention, cells that are used are preferably cells that can proliferate receptor ligand (factor)-dependently (cells with ligand (factor)-dependent proliferation). Preferably, on binding with a ligand, the receptors of the present invention usually transduce cell growth signals. However, when the receptors of the present invention are of a type that do not transduce cell growth signals, they can be used in the present invention as so-called "chimeric receptors", by fusing with receptors of a type that do transduce cell growth signals. More specifically, a chimeric receptor that comprises an extracellular region of a ligand-binding receptor, and an intracellular region of a type of receptor that transduces cell growth signals can be used. These chimeric receptors transduce cell growth signals on binding with a ligand. Receptors suitable for constructing chimeric receptors by fusion with ligand-binding receptors are not especially limited as long as they are of a type that transduces cell growth signals. Receptors that can be used include cytokine receptors and receptors known to those skilled in the art. G-CSF receptor,

mpl, neu, GM-CSF receptor, EPO receptor, c-Kit, FLT-3 and such are specific examples of such receptors. A suitable example of the above cells that grow ligand (factor)-dependently is a BaF3 ligand (factor)-dependent cell that expresses a chimeric receptor whose extracellular portion is a ligand receptor chain, and whose intracellular portion is a G-CSF receptor chain. Other examples of cells that can be used in the present invention include NFS60, FDCP-1, FDCP-2, CTLL-2, DA-1, KT-3, and such.

Please replace the paragraph beginning at page 12, line 30 with the following amended paragraph:

As described above, the present invention comprises antibodies, and cells that express receptors which multimerize on binding with those antibodies. The present invention also comprises cells whose growth is dependent on ~~those receptor~~ ligands (factors) against the receptor.

Please replace the paragraph beginning at page 16, line 11 with the following amended paragraph:

The antibody libraries can be constructed by known methods (see for example, McCafferty *et al.*, Nature 348: 55-554 (1990); Clackson *et al.*, Nature 352: 624-628 (1991); Marks *et al.*, J. Mol. Biol. 222: 582-597 (1991), etc). More specifically, antibody libraries can be constructed as follows, but the construction methods are not limited to these. First, mice are immunized with either A-chain or B-chain receptors, and mRNAs are extracted from the splenocytes of these animals. The L-chain and H-chain variable regions are then recovered by RT-PCR using primers that correspond to mice CDRs. Single chain variable regions (scFv) are synthesized using assembly PCR to construct antibody libraries. Antigen-binding antibody clones are concentrated by panning, these single chain variable regions obtained from concentrated clones are inserted between a signal sequence for animal cells and CH1-hinge-CH2-CH3 , and plasmids into which libraries are incorporated for generating retroviruses are

constructed. Alternatively, scFab is synthesized, and libraries inserted between a signal sequence and hinge-CH2-CH3 are constructed. scDb libraries can also be made.

Please replace the paragraph beginning at page 19, line 3 with the following amended paragraph:

In the above-mentioned screening methods of the present invention, whether or not a test antibody comprises agonistic ~~function~~ activity is then determined (step b), and antibodies comprising agonistic ~~function~~ activity are selected (step c).

Please replace the paragraph beginning at page 19, line 7 with the following amended paragraph:

In the above-mentioned steps in a preferable embodiment of the present invention, whether or not test antibodies comprise agonistic ~~function~~ activity is judged by using the presence or absence of the above-mentioned autonomous autocrine cell replication as an index. Where there is autonomous autocrine cell replication, antibodies expressed by those cells are selected as agonistic antibodies.

Please replace the paragraph beginning at page 19, line 31 with the following amended paragraph:

Next, a cell line that proliferates ligand-dependently is prepared. An anti-A-chian antibody library is introduced into these cells by infection, and infected cells are selected ~~by agent resistance, which was incorporated in the vector~~ using a drug-resistant gene incorporated into the vector. Selected cells are then cultured and subsequently, super-infected with the anti-B-chain antibody library. In this way, a library of cells expressing bispecific antibodies of every anti-A-chian antibody and anti-B-chian antibody combination can be constructed. Of these, only clones that secrete appropriately combined bispecific antibodies showing agonistic ~~function~~ activity against a target receptor can autonomously replicate upon autocrine stimulation. In this

way, the engineered antibody genes can be recovered by PCR from the chromosomes of selected BaF3 clones.

Please replace the paragraph beginning at page 20, line 8 with the following amended paragraph:

Methods of screening for antibodies that comprise agonistic ~~function~~ activity by using autonomous autocrine growth were unknown until now, and hence were newly discovered by the present inventors. The present invention provides agonistic antibody screening methods that use autonomous autocrine replication due to common antibodies, including multi-specific antibodies.

Please replace the paragraph beginning at page 20, line 14 with the following amended paragraph:

The above-mentioned screening methods first provide cells that express a test antibody and a receptor that multimerizes, where the growth of those cells depends on a ligand (factor) of that receptor (step A). Next, where the cells undergo autonomous autocrine replication, the test antibodies are judged to comprise agonistic ~~function~~ activity (step B). Antibodies that comprise agonistic ~~function~~ activity are then selected (step C).

Please replace the paragraph beginning at page 21, line 4 with the following amended paragraph:

In the above methods, agonistic antibodies are first screened by the methods of screening for agonistic antibodies of the present invention, and genes that code for the agonistic antibodies selected by this screening are introduced into host cells. Multi-specific agonistic antibodies are then recovered from those host cells or their culture supernatant.

Please replace the paragraph beginning at page 22, line 14 with the following amended paragraph:

Agonistic antibodies that can be acquired by the screening methods of the present invention can be used as pharmaceutical agents for immunotherapy or prevention, in the same way as traditionally known multi-specific antibodies. IL-10, 12, 24, 4, 7, 9, 13, TSLP, IFNα, β and such are known as ligands for immune system receptors made up of heterodimers. NGF, GDNF, NT-3, 4 and 5 are known as ligands for nervous system receptors. Antibodies acquired by the methods of the present invention can be, for example, antibodies that comprise an above-mentioned ligand-like function. The antibodies acquired by the methods of the present invention are expected to become pharmaceuticals for therapy of immune or nervous system illnesses and such.

Please replace the paragraph beginning at page 24, line 18 with the following amended paragraph:

Figure 1 is a graph showing Conditioned medium (CM)-dependent growth of an autonomously replicating cell line. 200μL of various concentrations of culture supernatants of MPL-expressing BaF3 cells that acquired autonomous replication by viral infection, was added to each well containing 10,000 washed MPL-expressing BaF3 cells (cells expressing chimeric receptors of the MPL extracellular region, and GCSF receptor transmembrane/intracellular region: TPG). After three days, 20μL of SF reagent (~~Nakarai~~ Nacarai Tesque) for measuring the viable cell number was added. After two and a half hours, absorbance was measured at 450 nm, and the number of viable cells was investigated. MPL-expressing BaF3 cells (HL(TPG) and LH(TPG)) maintained cell growth in a culture supernatant concentration-dependant manner. On the other hand, parent cell lines BaF3 (HL(BaF3) and LH(BaF3)), which do not express receptors, did not grow, since the antibodies do not act on these cells. The x-axis shows Conditioned medium (CM) concentration (%). The z-axis shows absorbance at 450-655 nm.

Please replace the paragraph beginning at page 25, line 5 with the following amended paragraph:

As a model of agonistic antibodies, the anti-mpl-monoclonal antibody 12E10 (WO99/10494) was used. An *Eco*RI-*Not*I fragment encoding a diabody was excised from pCOSscl2E10 (WO01/79494, WO02/33072) encoding the antibody 12E10 variable region. This fragment was inserted between the *Eco*RI and *Not*I of viral vector plasmid pMX. This plasmid pMXscl2E10 was transfected into Pt-E packaging cells using FuGene6 (Roche). Pt-E cells were seeded in 6 cm dishes containing Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum (FCS). The next day a mixture of FuGene6 and plasmid pMXsc12E10 was added to the culture. A day after that, the culture medium was replaced, and the culture supernatant was collected after 24 hours. 10μg/mL of Polybrene (Hexadimethrine Bromide, Sigma) was added to culture supernatant containing the recombinant virus, target cells were suspended in this culture supernatant and infected. Human MPL cDNA was introduced into ligand (factor)-dependent cell line BaF3. Cell line MPL/BaF3 (cells expressing chimeric receptors of the MPL extracellular region, and GCSF receptor transmembrane/intracellular region), which can grow due to the addition of MPL ligand (thrombopoietin), was infected by adding a viral solution with mouse interleukin-3 (IL-3). The next day the cells were washed with PBS. Culture was continued in ~~factor~~IL-3-free RPMI with 10% FCS, and autonomously replicating cells were obtained. Where the culture supernatant of these cells was collected and added to different MPL/BaF3 for incubation, a cell growth dependant on the concentration of the added culture supernatant was observed. From this it was clear that the autonomous replication of virus-infected cells was due to the autocrine stimulation of diabodies secreted into the culture medium by these cells.

Please replace the paragraph beginning at page 27, line 5 with the following amended paragraph:

The synthesized IgG1 constant region gene was inserted in the *Eco*RI-*Not*I site of a retroviral vector pMX, and pMX-CHwild was constructed. pMX-HL-CHwild was obtained by incorporating the scFv (H-L) gene into the *Eco*RI-*Sfi*I site of the pMX-CHwild plasmid. When the virus derived from this plasmid infects cells, molecules used as antibodies, which have CH1-hinge-CH2-CH3 linked with the scFV (H-L) C-terminal of antibody 12B5, can be expected to be

secreted. Then, IL3ss was incorporated into the *Bam*H1-*Eco*RI site of pMX-CHwild, and scFv(L-H) was further incorporated into the *Sfi*I site to construct pMX-IL3ss-LH-CHwild. ScFv-CH1-hinge-CH2-CH3 (L-H) can be expected to be secreted by this plasmid-derived virus. The above plasmids were respectively transfected into Pt-E cells, as above. Recombinant viruses were obtained and used to infect MPL/BaF3 cells. The next day, ~~factors~~ IL-3 ~~were~~ was removed by washing, and culture was continued. As a result, cells that can replicate autonomously were obtained. The culture supernatant of these cells was collected, and on culturing with different MPL/BaF3s, cell growth dependant on the concentration of culture supernatant added was observed (Figure 1, TPG). On the other hand, parent line BaF3 did not express the receptors, and thus did not proliferate as antibodies did not act on them (Figure 1, BaF3). From this, the autonomous replication of virus-infected cells was revealed to be due to the autocrine stimulation of scFv-CH1-hinge-CH2-CH3 secreted into the medium by those cells.